



OxLDL induced cell death is inhibited by the macrophage synthesised pterin, 7,8-dihydroneopterin, in U937 cells but not THP-1 cells

Sarah K. Baird^a, Linzi Reid^a, Mark B. Hampton^b, Steven P. Gieseg^{a,*}

^aFree Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

^bDepartment of Pathology, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand

Received 7 March 2005; received in revised form 4 July 2005; accepted 6 July 2005

Available online 18 July 2005

Abstract

The atherosclerotic plaque is an inflammatory site where macrophage cells are exposed to cytotoxic oxidised low density lipoprotein (oxLDL). Interferon- γ released from T-cells results in macrophage synthesis of 7,8-dihydroneopterin which has antioxidant and cytoprotective activity. Using the human derived monocyte-like U937 and THP-1 cell lines, we examined whether 7,8-dihydroneopterin could inhibit the cytotoxic effect of oxLDL. In U937 cells, oxLDL caused a dramatic loss of cellular glutathione and caspase independent cell death associated with phosphatidylserine exposure on the plasma membrane. 7,8-Dihydroneopterin completely blocked the cytotoxic effect of oxLDL. In contrast, oxLDL initiated THP-1 cell apoptosis with reduction in cellular thiols, caspase-3 activation and plasma membrane phosphatidylserine exposure. 7,8-Dihydroneopterin was unable to alter these processes or restore the THP-1 cellular thiol content. 7,8-Dihydroneopterin did provide some protection to both THP-1 cells and U937 cells from AAPH derived peroxy radicals. The preincubation of oxLDL with 7,8-dihydroneopterin did not reduce cytotoxicity, suggesting that 7,8-dihydroneopterin may be acting in U937 cells by scavenging intracellular oxidants generated by the oxLDL. The data show that μ M levels of 7,8-dihydroneopterin may prevent oxLDL mediated cellular death within atherosclerotic plaques.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Neopterin; Oxidised-low-density-lipoprotein; Macrophage; Caspase; Apoptosis; Glutathione

1. Introduction

The presence of immune cells and markers of immune cell activation strongly support the view that atherosclerotic plaques are sites of localised chronic inflammation [1–3]. One of the immune markers reported to correlate with atherosclerotic plaque development is plasma neopterin [4,5]. Neopterin can be formed through the oxidation of

7,8-dihydroneopterin by HOCl [6,7]. The presence of chloro-tyrosine indicates the generation of HOCl within atherosclerotic plaques and γ -interferon stimulation of macrophages results in the synthesis and release of 7,8-dihydroneopterin [8,9]. This would suggest that a probable source of the plasma neopterin is activated macrophages within atherosclerotic plaques.

The exact function of macrophage 7,8-dihydroneopterin synthesis and release in response to γ -interferon stimulation is uncertain. 7,8-Dihydroneopterin is a redox active compound capable of acting as either a pro-oxidant or antioxidant depending on the chemical environment. We have previously shown that at low μ M concentrations 7,8-dihydroneopterin is a potent antioxidant capable of protecting cells, cellular membranes [6], free proteins, cellular protein and protein thiols [10–12] from oxidant damage. Copper, peroxy radical and cell mediated LDL oxidation is

Abbreviations: AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid disodium salt; GSH, glutathione; HPLC, high performance liquid chromatography; LDL, Low density lipoprotein; oxLDL, oxidised low density lipoprotein; PS, phosphatidylserine; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate

* Corresponding author. Tel.: +64 3 364 2860; fax: +64 3 364 2024.

E-mail address: Steven.Gieseg@canterbury.ac.nz (S.P. Gieseg).

also effectively inhibited by μM levels of 7,8-dihydroneopterin [13–15]. Much of this protection is due to the potent radical scavenging ability of 7,8-dihydroneopterin with peroxyl radicals, superoxide, hypochlorite and hydroxyl radicals [12,16–18]. These observations have led us and others to suggest that 7,8-dihydroneopterin may be synthesised to protect macrophages from the oxidants present in inflammatory sites such as atherosclerotic plaques [12,13]. At mM pterin concentration neopterin and 7,8-dihydroneopterin have been reported to promote radical generation and apoptosis in a range of cell types exposed to different forms of cellular stress [19–22].

One of the major oxidants present within atherosclerotic plaques is oxidised LDL (oxLDL) [23]. The uncontrolled uptake of oxLDL by macrophages results in the formation of lipid loaded foam cells. It is the build up of foam cells within the artery wall which characterises the atherosclerotic plaque [24]. The cytotoxicity of oxLDL appears to cause the death of macrophages and smooth muscle cells deep within the plaque, leading to the formation of a necrotic core region [23,25,26].

To provide some insight into the possible effect of 7,8-dihydroneopterin within plaques, we have examined the effect of this pterin in two different human monocyte-like cell lines exposed to oxLDL. The THP-1 and U937 cell lines have been extensively used to model aspects of atherosclerosis and are equally susceptible to oxLDL induced cell death. We have previously reported that oxLDL triggers caspase-3 activation and apoptotic cell death in THP-1 cells, while cell death in the U937 cells occurs independently of caspase-3 activity and is characterised by a massive loss of intracellular thiols [27].

2. Methods

All chemicals used were of AR grade or better and supplied by either the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited. Tissue culture media including the heat inactivated foetal calf serum was supplied by Invitrogen New Zealand Limited and plastic ware from Sarstedt Australia Pty Ltd. through Global Science and Technology Ltd. (New Zealand). Neopterin and 7,8-dihydroneopterin were obtained from Schirck's Laboratories, Switzerland. 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company Inc. Chelex-100 resin was supplied by Bio-Rad Laboratories (NZ). All solutions were prepared using ion-exchanged ultrafiltered water from a NANOpure ultrapure water system supplied by Barnstead/Thermolyne (Iowa, USA). Phosphate buffered saline (PBS) solution consisted of 150 mM sodium chloride and 10 mM sodium phosphate pH 7.4.

LDL was purified from EDTA treated plasma collected by venapuncture from healthy male and female donors following an overnight fast. After removal of cellular

components by centrifugation, the plasma from multiple donors was pooled and frozen at -80°C for up to 6 months. LDL was purified by a single 22 h ultracentrifugation of the pooled EDTA-plasma using a buoyant density four step discontinuous gradient in a Beckman SW41 rotor [28,29]. LDL concentration (total mass) was determined by enzymatic cholesterol determination using the "Chol MPR 2" kit supplied by Roche Chemicals (New Zealand), assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6% [29]. Purified LDL was desalted by dialysis against nitrogen-gassed chelex-treated phosphate buffered saline (pH 7.4). OxLDL was prepared by incubating LDL at a concentration of 4 mg/ml with 200 μM CuSO_4 solution for 24 h at 37°C before stirring with chelex-100 resin for 2 h to remove copper ions. HPLC analysis [14] showed the oxidation had increased the LDL TBARS level from 0.2 to 1.9 nmol/mg LDL. The electrophoretic mobility of the oxLDL was 3.2-fold higher than the native LDL. Native and oxLDL were concentrated with Urifil-10 concentrators (Millipore, MA, USA) and sterilised through a 0.22- μm membrane filter (Pall Gelman Laboratory) before addition to the cells.

THP-1 and U937 cells were grown in RPMI 1640 with 2 mM glutamine, 5% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, in a humidified atmosphere with 5% CO_2 at a density of not more than $2 \times 10^6/\text{ml}$. During experiments, cells were incubated for up to 48 h in RPMI 1640 alone at 5×10^5 cells/ml with the described additives before washing in warm PBS to remove the media before analysis.

Cell viability was determined by trypan blue dye exclusion [30] and the cells' ability to reduce MTT (Sigma) [31] by using 10% w/v sodium dodecyl sulphate (SDS, final concentration) to lyse the cells and solubilise the insoluble MTT-formazan salt.

Total cellular thiol content was determined by incubating the PBS washed cells in 10% w/v SDS and 30 μM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, final concentration shown) for 30 min at room temperature and reading the absorbance at 412 nm [32].

Cellular glutathione (GSH) was measured in PBS washed cells by derivatisation with monobromobimane and analysis by gradient reverse phase high performance liquid chromatography (HPLC) with fluorescence detection [33]. The GSH concentration was calculated from comparison to peak area of known concentrations of glutathione solutions, standardised using DTNB.

7,8-Dihydroneopterin and neopterin concentrations in the media were measured by reverse phase HPLC with electrochemical and fluorescence detection as previously described [34].

Caspase-3-like activity was determined by measuring the rate of cleavage of the artificial substrate DEVD-AMC (Sigma) [35]. PBS washed cells (1×10^6) were lysed in 100 μL of caspase reagent buffer containing 100 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid),

10% w/v sucrose, 0.1% v/v CHAPS (3-[(3-chloramido-propyl) dimethylammonio]-1-propane sulphonate), $10^{-4}\%$ v/v NP-40, 5 mM DTT and 50 μ M DEVD-AMC (acetyl-aspglu-val-asp-7-amido-4-methyl-coumarin) pH 7.25. The Caspase-3 activity was determined measuring the increase in fluorescence over time in a heat-jacketed F-4500 Hitachi fluorometer at λ_{ex} , 370 nm and λ_{em} , 445 nm at 37 °C. Fluorescence units were converted to pmol of free AMC using a standard curve generated with reagent AMC.

Cells were pelleted and suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI), as per the manufacturer's instructions (Apoptest-FITC A700 kit, Nexins Research). Following ten min in the dark, 10,000 cells were analysed with a Vantage fluorescence-activated cell sorter from Becton Dickinson (San Jose, CA). The cells were classified as viable, apoptotic or necrotic based on regions drawn on the dot plots. Cells with low Annexin V-FITC and PI fluorescence were classified as viable, cells binding Annexin V-FITC but excluding PI were classified as apoptotic and double labelled cells were classified as necrotic [36].

The data were analysed using the Prism software package supplied by Graphpad Software Inc. Comparisons among treatments were performed using one-way analysis of variance (ANOVA). When significance was observed ($P \leq 0.05$), Tukey's multiple comparison test was performed to determine which means differed from the control by a significant margin. All results are expressed as the mean \pm S.D. of triplicate treatments. Results shown are from single experiments, representative of a minimum of three. Where appropriate on figures significance is indicated as *** $P \leq 0.001$; ** $P \leq 0.01$, * $P \leq 0.05$.

3. Results

Incubation of THP-1 and U937 cells with 1.5 mg/ml oxLDL caused similar losses in cell viability, as measured by the cells ability to exclude the dye trypan blue or reduce MTT (Fig. 1). The addition of 7,8-dihydroneopterin to U937 cells reduced this loss of oxLDL mediated cell viability (Fig. 1A). The same trend was seen with both cell viability assays even though they measure very different aspects of cell activity.

In contrast, the incubation of THP-1 cells with 7,8-dihydroneopterin failed to prevent the loss of cell viability as measured by either assay. The addition of 7,8-dihydroneopterin in the absence of oxLDL had no significant effect on the viability of either cell type. Studies with up to 200 μ M neopterin failed to show any protective effect against oxLDL with either THP-1 or U937 cells.

Under light microscopy, the effect of the oxLDL treatment on the U937 cells could be clearly seen with the loss of normal cell morphology and the appearance of many shrunken and distorted cells (Fig. 2). Increasing the concentration of 7,8-dihydroneopterin prevented this dam-

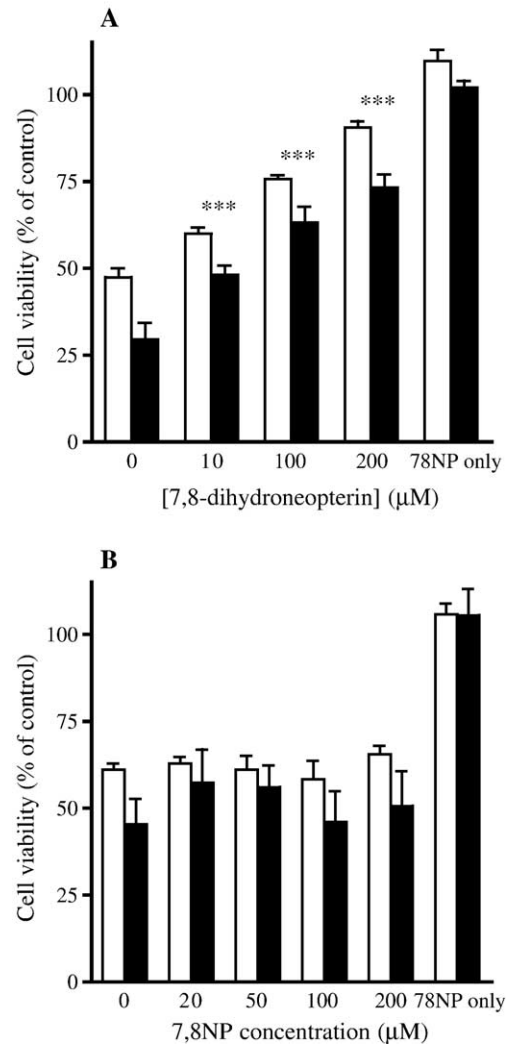


Fig. 1. 7,8-Dihydroneopterin protects U937 but not THP-1 cells from oxLDL induced loss of cell viability. U937 (A) or THP-1 cells (B) were suspended at 5×10^5 cells/ml in RPMI 1640 with 1.5 mg/ml oxLDL and increasing concentrations of 7,8-dihydroneopterin (78 NP). After 48 h, the cell viability was measured by the MTT reduction assay (white bars) and the trypan blue dye exclusion assay (black bars). A control incubation of cells with 200 μ M 7,8-dihydroneopterin (labelled 78 NP only) was included to show 7,8-dihydroneopterin did not affect cell viability. Data are expressed as a percentage of the value obtained from cells which had no oxLDL added.

age with the 200 μ M 7,8-dihydroneopterin treated cells appearing morphologically similar to the untreated control. Treatment with 200 μ M 7,8-dihydroneopterin alone had no effect on the cell morphology. OxLDL treatment of the THP-1 cells resulted in a similar loss of normal cell morphology with the appearance of many shrunken and lysed cells as previously described [27]. Treatment with 7,8-dihydroneopterin, even at 200 μ M failed to prevent the loss of the normal THP-1 cell morphology (data not shown).

The loss of cell viability and the effect of 7,8-dihydroneopterin was further characterised by flow cytometry analysis of phosphatidylserine externalization and cell lysis. OxLDL caused a 57% loss of normal U937 cells (Fig.

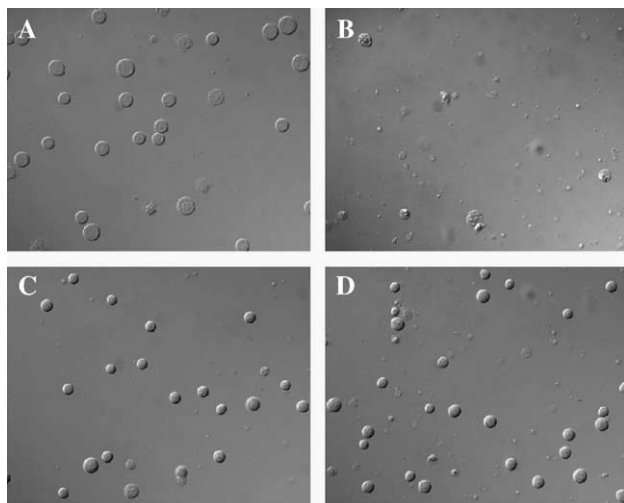


Fig. 2. 7,8-Dihydroneopterin prevents loss of U937 cell morphology. U937 cells were suspended at 5×10^5 cells/ml in RPMI 1640 with 1.5 mg/ml oxLDL and increasing concentrations of 7,8-dihydroneopterin for 48 h before being photographed under a light microscope. (A) Control without oxLDL; (B) oxLDL; (C) oxLDL+200 μ M 7,8-dihydroneopterin; (D) 200 μ M 7,8-dihydroneopterin no oxLDL.

3A) which is in relatively good agreement with the cell viability assays (Fig. 1A). Of these abnormal U937 cells, the majority became necrotic as shown by positive staining by both annexin-V and PI. A smaller rise in apparent apoptotic cells is also seen. Treatment with 7,8-dihydroneopterin significantly reduced ($P < 0.001$) the size of this necrotic population to almost half of its previous level with a corresponding doubling in the number of normal cells (Fig. 3A and E). Though this increase with 7,8-dihydroneopterin was highly significant, the number of normal cells was not restored to that seen with the controls. In contrast, 7,8-dihydroneopterin did restore MTT reduction activity to 90% of the untreated control cells (Fig. 1A), possibly due to metabolic activity of annexin V staining apoptotic cells which made up 30% of the oxLDL treated U937 cell population.

THP-1 cells were treated with 1 mg/ml oxLDL, which we have previously shown causes the cells to become apoptotic [27] (Fig. 3B). Treatment with 7,8-dihydroneopterin resulted in a small but insignificant increase in normal cells. Similarly, 7,8-dihydroneopterin had no effect on caspase-3 activity in the THP-1 cells (Fig. 4). In the absence of oxLDL, 7,8-dihydroneopterin did not activate caspase-3 in THP-1 cells. With U937 cells, no caspase-3 activity was observed in response to oxLDL treatment, with or without 7,8-dihydroneopterin addition (data not shown). Neopterin had no effect on either cell line in the presence or absence of oxLDL.

Our previous studies had shown that 1.5 mg/ml oxLDL causes a massive 90% loss of intracellular thiols in U937 cells [27]. The addition of 7,8-dihydroneopterin resulted in concentration dependent protection of the cellular thiol pool with 200 μ M 7,8-dihydroneopterin reducing the oxLDL

mediated loss to only 50% of the control cells' levels (Fig. 5). The increase in cellular thiols was not significant below 100 μ M 7,8-dihydroneopterin. The DTNB assay measures all cellular thiol groups so using a separate HPLC method we measured the changes in the U937 GSH levels. OxLDL completely eliminated the GSH levels but 100 μ M 7,8-dihydroneopterin reduced this to only 40% compared to the control while 200 μ M increased the GSH level to a concentration significantly higher than the control ($P < 0.001$). In contrast, oxLDL treatment only caused a significant ($P < 0.001$) 25% loss in the THP-1 cell thiol content (Fig. 5B). The THP-1 thiol loss could not be prevented by the addition of 7,8-dihydroneopterin. The addition of native LDL to either cell line did not significantly alter the intracellular thiol levels (data not shown).

OxLDL was not immediately toxic to the U937 cells but required a minimum of 6 h of exposure before a significant loss in cell viability was observed. We found that if the oxLDL was removed after less than 6 h and the cells incubated for a further 36 h, only a 25% loss in MTT reducing activity was observed (data not shown). If the oxLDL exposure was increased to 12 h before changing the media the loss in MTT reducing activity was increased to 75%. It is during this 6 h window that 7,8-dihydroneopterin was found to be effective at protecting the U937 cells from oxLDL toxicity. 7,8-Dihydroneopterin could be added up to 6 h after the oxLDL and still provided a significant 8% increase ($P < 0.1$) in U937 cell viability (Fig. 6). We found there was no difference between adding the 7,8-dihydroneopterin with the oxLDL or 2 h later. Both treatments provide a 27% increase in MTT reducing activity compared to the oxLDL treated control cells. This time window during which 7,8-dihydroneopterin can be added to provide protection to cells is in marked contrast to our previous studies which found that cells and lipoproteins need to be preincubated for up to 10 min before oxidant addition to gain maximum protection [10,12,13]. Preincubation of THP-1 cells with 200 μ M 7,8-dihydroneopterin up to 2 h before oxLDL addition still failed to prevent the loss of cell viability.

Though the data indicated that the 7,8-dihydroneopterin was causing some change in the intracellular environment of the U937 cells we did examine whether incubating the oxLDL with 7,8-dihydroneopterin could reduce the cellular toxicity. This analysis showed that 7,8-dihydroneopterin had no direct effect on oxLDL chemical toxicity (data not shown), further supporting the idea that 7,8-dihydroneopterin is acting on the cells rather than the oxLDL.

The striking difference in the effect of 7,8-dihydroneopterin on the two cell types lead us to examine the cells' susceptibility to peroxy radicals generated by the thermolysis of AAPH. As with previous studies [12], 7,8-dihydroneopterin was found to effectively scavenge peroxy radicals so reducing the loss in cell viability (Fig. 7A).

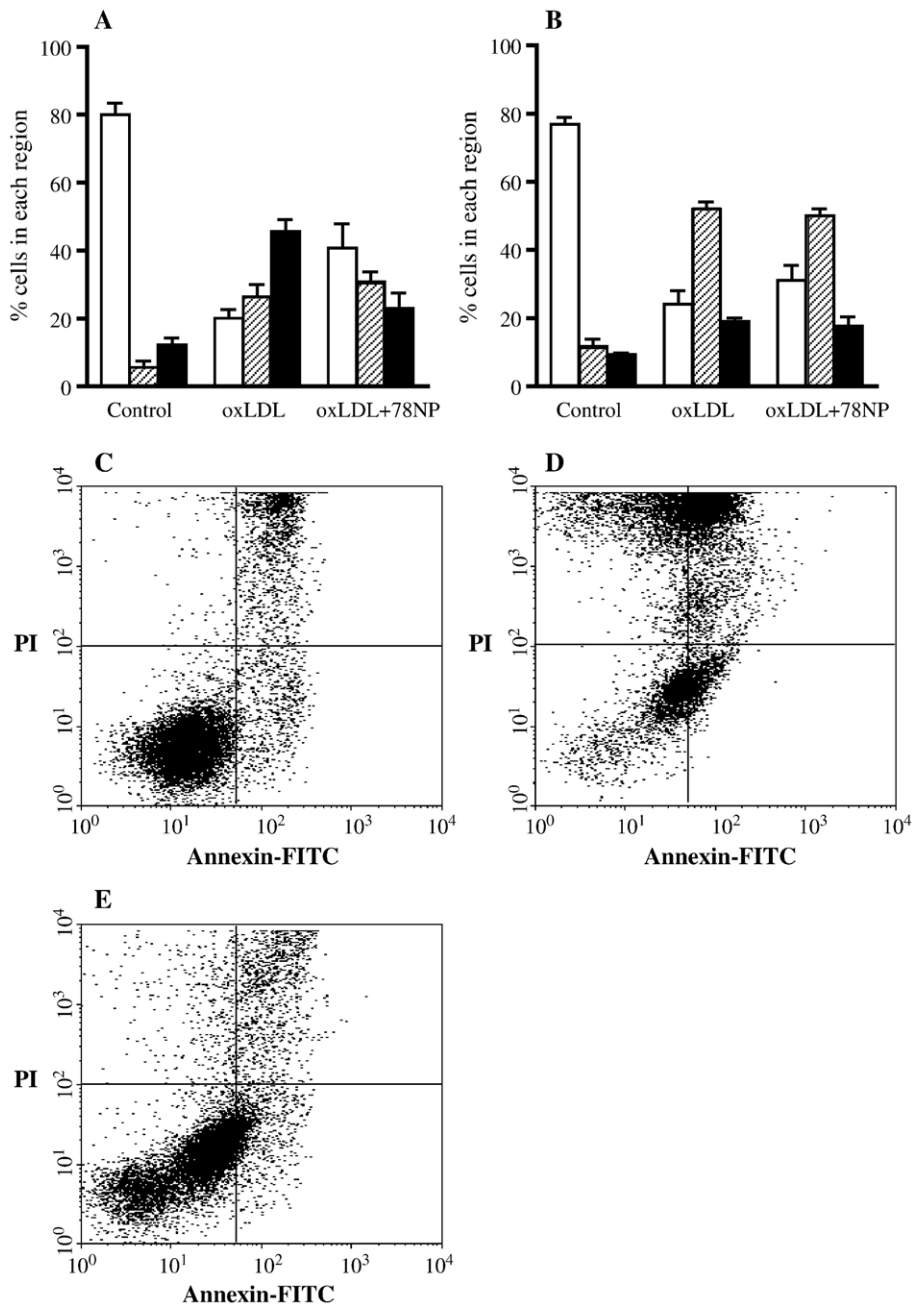


Fig. 3. 7,8-Dihydroneopterin reduced phosphatidylserine exposure on oxLDL treated U937 cells but not THP-1 cells. U937 (A) or THP-1 (B) cells were incubated for 48 h at 5×10^5 /ml in RPMI 1640 with a combination of either of 200 μ M 7,8-dihydroneopterin (78 NP) or oxLDL. U937 cells were treated with 1.5 mg/ml oxLDL and THP-1 cells with 1 mg/ml oxLDL. The cells were co-stained with annexin V-FITC and propidium iodide, and the fluorescent labelling assessed on a FACS vantage flow cytometer. The open bars represent normal cells, hatched bars the apoptotic cells and solid bars the necrotic cells. A representative experiment showing 5000 U937 cells treated consisting of untreated U937 cells (C); U937 cells incubated with 1.5 mg/ml oxLDL (D); or U937 cells incubated with both 1.5 mg/ml oxLDL and 200 μ M 7,8-dihydroneopterin (E); is presented.

Surprisingly, AAPH caused a similar loss in cellular thiols in both cell types (approximately 40%) but it was only in the U937 cells that 7,8-dihydroneopterin was able to prevent the loss in total cellular thiols (Fig. 7B). Although oxLDL and AAPH appear to cause very different forms of oxidative stress, 7,8-dihydroneopterin was only able to protect the thiols in the U937 cells (Figs. 5 and 7). No significant difference in the total thiol

concentration was found between the control THP-1 and U937 cells.

HPLC analysis of the endogenous pterin concentrations showed the level of 7,8-dihydroneopterin was too low to cause a significant cellular difference between the two cell lines. Media from THP-1 cells had a pterin concentration (neopterin+7,8-dihydroneopterin) of 25.5 nM while media incubated with U937 cells had a concentration of 16.58 nM.

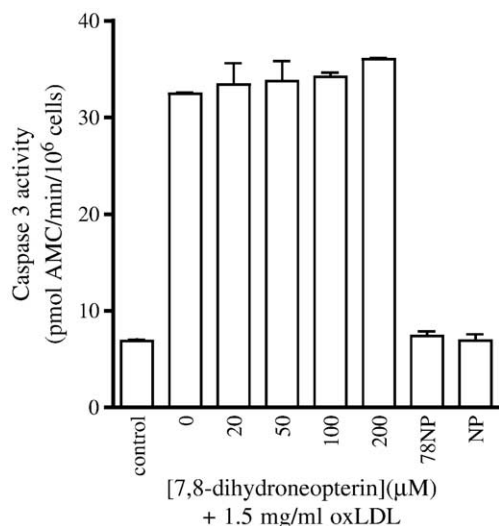


Fig. 4. 7,8-Dihydroneopterin had no effect on caspase-3 activation in oxLDL treated THP-1 cells. THP-1 cells at 5×10^5 cells/ml were suspended in RPMI 1640 to which was added 1.5 mg/ml oxLDL, with increasing concentrations of 7,8-dihydroneopterin. The control group was cells with no oxLDL or 7,8-dihydroneopterin added. Two additional controls involved cells with no added oxLDL but containing either 200 μ M 7,8-dihydroneopterin (78 NP) or 200 μ M neopterin (NP). After 48 h of incubation, the cells were harvested and the caspase-3 like activity measured by monitoring the hydrolysis of DEVD-AMC.

There was no significant difference in intracellular concentration with U937 cells having 2.8 nmol pterin/mg protein while the THP-1 intracellular concentration was 2.7 pterin nmol/mg protein.

4. Discussion

The regulation of cellular GSH levels appears to be a key component determining the fate of oxidatively stressed cells. In U937 cells, the rapid thiol loss induced by oxLDL resulted in caspase independent cellular death which appeared more necrotic than apoptotic in nature. Caspase enzymes are sensitive to cellular redox states as the active site includes a reduced cysteine thiol residue. Oxidation of the caspase thiol results in the loss of caspase activity [37]. The total loss of the U937 GSH and protein thiols would have greatly increased the intracellular oxidative stress leading to the observed loss of caspase activity. Macrophage like RAW264.7 cells treated with BSO have also been reported to lose caspase activity during nitric oxide donor initiated apoptosis [38]. The failure of the caspase apoptosis system may have been responsible in part for the necrotic nature of the oxLDL-treated U937 cells when examined by flow cytometry (Fig. 3).

In contrast, the THP-1 cells' thiol levels were not as severely affected by the oxLDL (Fig. 5B) even though both cell lines have a similar sensitivity to oxLDL induced loss of cell viability [27]. The level of cellular thiols was maintained at 75% of untreated THP-1 cells and caspase-3 was activated during the apoptosis.

We have previously shown 7,8-dihydroneopterin to be a good inhibitor of various forms of oxidative damage to cells [10,12]. It was therefore surprising to find such a striking difference in the level of protection afforded by 7,8-dihydroneopterin between the two cell lines. 7,8-Dihydroneopterin failed to prevent oxLDL induced reduction in cellular thiols in the THP-1 cells and the initiation of apoptosis, whereas 200 μ M 7,8-dihydroneopterin completely restored cellular GSH levels in the U937 cells, with viability being returned to 75% of the control value (Figs. 1A and 5A).

The fact that 7,8-dihydroneopterin was observed to provide some protection to both cell lines exposed to

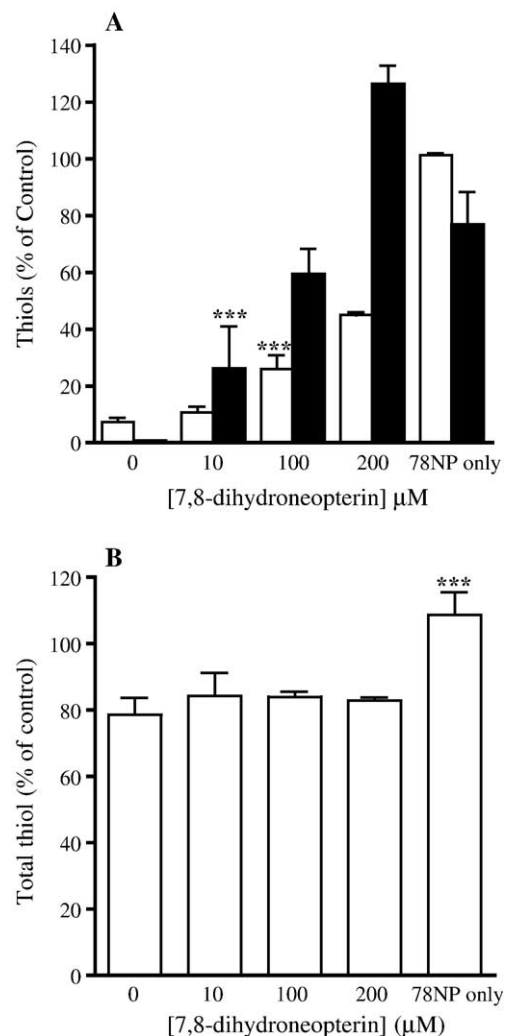


Fig. 5. 7,8-Dihydroneopterin increased intracellular thiol levels in oxLDL treated U937 but not THP-1 cells. U937 (A) or THP-1 cells (B) were suspended at 5×10^5 cells/ml in RPMI 1640 with 1.5 mg/ml oxLDL and increasing concentrations of 7,8-dihydroneopterin (78 NP). After 48 h the total cellular thiol content was measured using the DTNB assay and the values shown as white bars. The GSH concentration (black bars) was determined only for the U937 cells, by derivatisation with monobromobimane and HPLC analysis. A control incubation of cells with 200 μ M 7,8-dihydroneopterin (labelled 78 NP only) was included to show 7,8-dihydroneopterin did not cause thiol loss. Data are expressed as a percentage of the value obtained from cells which had no oxLDL added.

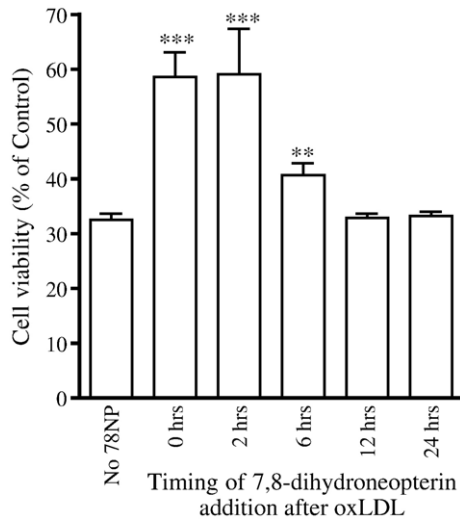


Fig. 6. 7,8-Dihydroneopterin inhibited the loss of cell viability up to 6 h after the addition of oxLDL to U937 cells. U937 cells were suspended in RPMI 1640 media containing 1.5 mg/ml oxLDL and 200 μ M 7,8-dihydroneopterin except for the control which had no 7,8-dihydroneopterin added. The 7,8-dihydroneopterin was added either at 0, 2, 6, 12 or 24 h after the oxLDL addition. Cell viability was measured by MTT reduction 48 h after the addition of the oxLDL. Data are expressed as a percentage of the value obtained from cells which had no oxLDL added.

AAPH peroxyl radicals suggests that the protection from oxLDL was not due to the scavenging of extracellular radicals or oxidants on the oxLDL (Fig. 7A). Preincubation of oxLDL with ascorbate or dehydroascorbate has been reported to inhibit oxLDL induced macrophage apoptosis, possibly by reacting with reactive groups on the ApoB100 [39]. With 7,8-dihydroneopterin, preincubation with oxLDL failed to reduce the level of viability loss showing that with U937 cells 7,8-dihydroneopterin is acting on a cellular mechanism. Trolox has been reported to inhibit oxLDL induced apoptosis in blood derived macrophages by scavenging intracellular peroxyl radicals [40]. α -Tocopherol, another peroxyl radical scavenger, has also been reported to protect U937 cells from 7 β -hydroxycholesterol induced GSH loss and apoptosis. As 7,8-dihydroneopterin is also a peroxyl radical scavenger, it is possible that a similar mechanism is occurring with the U937 cells. N-acetylcysteine and GSH have been reported to inhibit oxLDL and 7-ketocholesterol induced apoptosis by increasing the intracellular thiol pool in macrophages and U937 cells [41,42]. It is unlikely that 7,8-dihydroneopterin can regenerate or increase intracellular GSH levels as 7,8-dihydroneopterin is unable to reduce oxidised protein thiols [12]. This strongly suggests that with U937 cells, 7,8-dihydroneopterin is protecting the intracellular thiol pool by scavenging reactive oxygen species generated by the presence of oxLDL. A number of the lipid oxides found in oxLDL have been shown to increase the level of intracellular reactive oxygen species in macrophages [42,43]. How 7,8-dihydroneopterin reaches these intracellular reactive species and why this does not occur

with THP-1 cells is difficult to determine with the current data.

The results presented here clearly show that for some cell types and oxidative stresses, 7,8-dihydroneopterin is an anti-necrotic agent. This may appear to be in conflict with other studies showing 7,8-dihydroneopterin and neopterin inducing apoptosis or oxidative stress, but these studies use mM concentrations of the pterins [19–22]. Clearly, below 500 μ M 7,8-dihydroneopterin is a radical scavenger and inhibitor of cellular necrosis.

Assuming macrophage derived foam cells within the artery wall behave in a similar fashion to U937 cells, 7,8-dihydroneopterin can be expected to play a major role in the control of both oxLDL formation [14] and foam cell

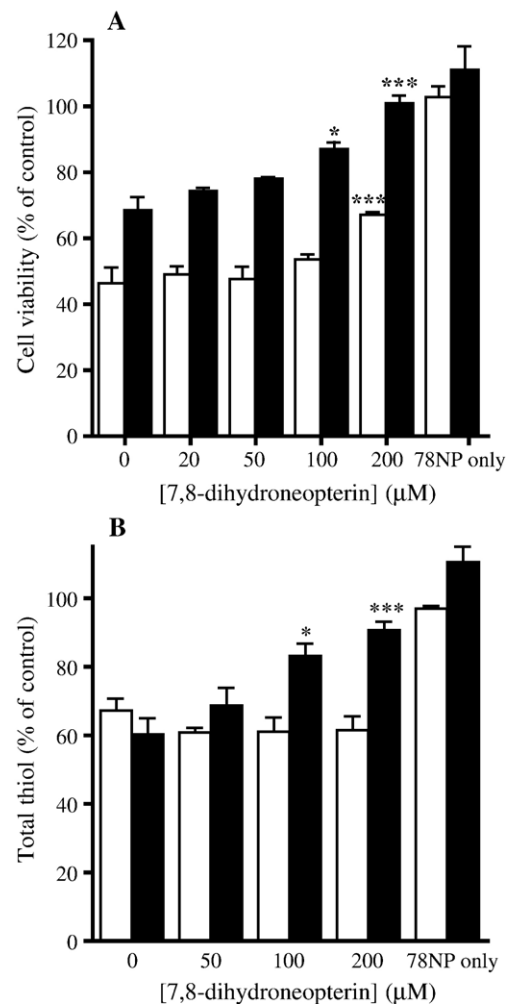


Fig. 7. The effect of 7,8-dihydroneopterin on AAPH treated cells. THP-1 (white bars) or U937 (black bars) were suspended at 5×10^5 cells/ml in Earle's balance salt solution with 10 mM AAPH and increasing concentrations of 7,8-dihydroneopterin. After 12 h, the cell viability was measured by MTT reduction analysis (A) and the total cellular thiol content (B) was measured using the DTNB thiol assay. A control incubation of cells with only 200 μ M 7,8-dihydroneopterin (labelled 78 NP only) was included to show 7,8-dihydroneopterin by itself did not cause thiol loss. Data are expressed as a percentage of the value obtained from cells which had no oxLDL added.

viability. Our preliminary evidence suggests that with blood derived macrophages exposed to oxLDL 7,8-dihydroneopterin inhibits apoptosis (unpublished results). The key question then becomes whether 7,8-dihydroneopterin can reach μM concentrations within atherosclerotic plaques to affect the apoptotic and scavenging activity observed in tissue culture. Though there is a considerable amount of literature on pterin levels in plasma and urine, the actually concentration within sites of inflammation is not known and therefore requires investigation.

Acknowledgements

This work was supported by a Project Grant from the National Heart Foundation of New Zealand. Sarah Baird was supported by a University of Canterbury Ph.D. Scholarship and Dr. Reid by a University of Canterbury Post Doctoral fellowship. We would especially like to thank Dr. Jinny Willis from the Lipid and Diabetes Research Group, Christchurch Hospital for taking the blood.

References

- [1] P. Libby, P.M. Ridker, A. Maseri, Inflammation and atherosclerosis, *Circulation* 105 (2002) 1135–1143.
- [2] M. Erren, H. Reinecke, R. Junker, M. Fobker, H. Schulte, J.O. Schurek, J. Kropf, S. Kerber, G. Breithardt, G. Assmann, P. Cullen, Systemic inflammatory parameters in patients with atherosclerosis of the coronary and peripheral arteries, *Arterioscler. Thromb. Vasc. Biol.* 10 (1999) 2355–2363.
- [3] W. Martinet, M.M. Kockx, Apoptosis in atherosclerosis: focus on oxidized lipids and inflammation, *Curr. Opin. Lipidol.* 12 (2001) 535–541.
- [4] M. Schumacher, G. Halwachs, F. Tatzber, F.M. Fruhwald, R. Zweiker, N. Watzinger, B. Eber, M. Wilders-Truschnig, H. Esterbauer, W. Klein, Increased neopterin in patients with chronic and acute coronary syndromes, *J. Am. Coll. Cardiol.* 30 (1997) 703–707.
- [5] F. Tatzber, H. Rabl, K. Koriska, U. Erhart, H. Puhl, G. Weag, A. Krebs, H. Esterbauer, Elevated serum neopterin levels in atherosclerosis, *Atherosclerosis* 89 (1991) 203–208.
- [6] S.P. Gieseg, G. Maghzal, D. Glubb, Inhibition of haemolysis by the macrophage synthesized antioxidant, 7,8-dihydroneopterin, *Redox Rep.* 5 (2000) 97–100.
- [7] B. Widner, C. Mayr, B. Wirleitner, D. Fuchs, Oxidation of 7,8-dihydroneopterin by hypochlorous acid yields neopterin, *Biochem. Biophys. Res. Commun.* 275 (2000) 307–311.
- [8] S.L. Hazen, J.W. Heinecke, 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima, *J. Clin. Invest.* 99 (1997) 2075–2081.
- [9] H. Wachter, D. Fuchs, A. Hausen, G. Reibnegger, E.R. Werner, Neopterin as marker for activation of cellular immunity: immunologic basis and clinical application, *Adv. Clin. Chem.* 27 (1989) 81–141.
- [10] S.P. Gieseg, J. Whybrow, D. Glubb, C. Rait, Protection of U937 cells from free radical damage by the macrophage synthesized antioxidant 7,8 dihydroneopterin, *Free Radic. Res.* 35 (2001) 311–318.
- [11] S. Duggan, C. Rait, J.M. Gebicki, S.P. Gieseg, Inhibition of protein oxidation by the macrophage synthesised antioxidant 7,8-dihydroneopterin, *Redox Rep.* 6 (2001) 188–190.
- [12] S.P. Gieseg, S. Duggan, C. Rait, A. Platt, Protein and thiol oxidation in cells exposed to peroxy radicals, is inhibited by the macrophage synthesised pterin 7,8-dihydroneopterin, *Biochim. Biophys. Acta* 1591 (2002) 139–145.
- [13] S.P. Gieseg, G. Reibnegger, H. Wachter, H. Esterbauer, 7,8-Dihydroneopterin inhibits low density lipoprotein oxidation in vitro. Evidence that this macrophage secreted pteridine is an anti-oxidant, *Free Radic. Res.* 23 (1995) 123–136.
- [14] S.P. Gieseg, S. Cato, Inhibition of THP-1 cell-mediated low-density lipoprotein oxidation by the macrophage-synthesised pterin, 7,8-dihydroneopterin, *Redox Rep.* 8 (2003) 113–119.
- [15] S.P. Gieseg, J. Pearson, C.A. Firth, Protein hydroperoxides are a major product of low density lipoprotein oxidation during copper, peroxy radical and macrophage-mediated oxidation, *Free Radic. Res.* 37 (2003) 983–991.
- [16] K. Watanabe, T. Arai, H. Mori, S. Nakao, T. Suzuki, K. Tajima, K. Makino, K. Mori, Pterin-6-aldehyde, an inhibitor of xanthine oxidase, has superoxide anion scavenging activity, *Biochem. Biophys. Res. Commun.* 233 (1997) 447–450.
- [17] G. Weiss, D. Fuchs, A. Hausen, G. Reibnegger, E.R. Werner, G. Werner-Felmayer, E. Semenitz, M.P. Dierich, H. Wachter, Neopterin modulates toxicity mediated by reactive oxygen and chloride species, *FEBS Lett.* 321 (1993) 89–92.
- [18] K. Oettl, S. Dikalov, H.J. Freisleben, W. Mlekusch, G. Reibnegger, Spin trapping study of antioxidant properties of neopterin and 7,8-dihydroneopterin, *Biochem. Biophys. Res. Commun.* 234 (1997) 774–778.
- [19] K. Oettl, G. Reibnegger, Pteridine derivatives as modulators of oxidative stress, *Curr. Drug Metab.* 3 (2002) 203–209.
- [20] C. Enzinger, B. Wirleitner, N. Spottl, G. Bock, D. Fuchs, G. Baier-Bitterlich, Reduced pteridine derivatives induce apoptosis in PC12 cells, *Neurochem. Int.* 41 (2002) 71–78.
- [21] G. Baier-Bitterlich, D. Fuchs, C. Murr, G. Reibnegger, G. Werner-Felmayer, R. Sgonc, G. Bock, M.P. Dierich, H. Wachter, Effect of neopterin and 7,8-dihydroneopterin on tumor necrosis factor- α induced programmed cell death, *FEBS Lett.* 364 (1995) 234–238.
- [22] C. Enzinger, B. Wirleitner, C. Lutz, G. Bock, B. Tomaselli, G. Baier, D. Fuchs, G. Baier-Bitterlich, 7,8-Dihydroneopterin induces apoptosis of Jurkat T-lymphocytes via a Bcl-2-sensitive pathway, *Eur. J. Cell Biol.* 81 (2002) 197–202.
- [23] R. Salvayre, N. Auge, H. Benoist, A. Negre-Salvayre, Oxidized low-density lipoprotein-induced apoptosis, *Biochim. Biophys. Acta* 1585 (2002) 213–221.
- [24] D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo, J.L. Witztum, Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity, *N. Engl. J. Med.* 320 (1989) 915–924.
- [25] L. Hegyi, J.N. Skepper, N.R.B. Cary, M.J. Mitchinson, Foam cell apoptosis and the development of the lipid core of human atherosclerosis, *J. Pathol.* 180 (1996) 423–429.
- [26] S.J. Hardwick, L. Hegyi, K. Clare, N.S. Law, K.L.H. Carpenter, M.J. Mitchinson, J.N. Skepper, Apoptosis in human monocyte-macrophages exposed to oxidised low density lipoprotein, *J. Pathol.* 179 (1996) 294–302.
- [27] S.K. Baird, M. Hampton, S.P. Gieseg, Oxidised LDL triggers phosphatidylserine exposure in human monocyte cell lines by both caspase-dependent and independent mechanisms, *FEBS Lett.* 578 (2004) 169–174.
- [28] B.H. Chung, J.P. Segrest, M.J. Ray, J.D. Brunzell, J.E. Hokanson, R.M. Krauss, K. Beaudrie, J.T. Cone, Single vertical spin density gradient centrifugation, *Methods Enzymol.* 128 (1986) 181–209.
- [29] S.P. Gieseg, H. Esterbauer, Low density lipoprotein is saturable by pro-oxidant copper, *FEBS Lett.* 343 (1994) 188–194.
- [30] P. Moldeus, J. Hogberg, S. Orrenius, Isolation and use of liver cells, *Methods Enzymol.* 52 (1978) 60–71.
- [31] T. Mosmann, Rapid colorimetric assay for cellular growth and

- survival: application to proliferation and cytotoxicity assays, *J. Immunol. Meth.* 65 (1983) 55–63.
- [32] M. Hu, Measurement of protein thiol groups and glutathione in plasma, *Methods Enzymol.* 233 (1994) 380–385.
- [33] I.A. Cotgreave, P. Moldeus, Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems, *J. Biochem. Biophys. Methods* 13 (1986) 231–249.
- [34] S.P. Gieseg, D. Glubb, G. Maghzal, Protection of erythrocytes by the macrophage synthesized antioxidant 7,8 dihydroneopterin, *Free Radic. Res.* 34 (2001) 123–136.
- [35] M.B. Hampton, I. Stamenkovic, C.C. Winterbourn, Interaction with substrate sensitises caspase-3 to inactivation by hydrogen peroxide, *FEBS Lett.* 517 (2002) 229–232.
- [36] I. Vermes, C. Haanen, H. Steffensnacken, C. Reutelingsperger, A novel assay for apoptosis-flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein-labeled annexin-V, *J. Immunol. Methods* 184 (1995) 39–51.
- [37] M.B. Hampton, S. Orrenius, Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis, *FEBS Lett.* 414 (1997) 552–556.
- [38] S.E. Boggs, T.S. McCormick, E.G. Lapetina, Glutathione levels determine apoptosis in macrophages, *Biochem. Biophys. Res. Commun.* 247 (1998) 229–233.
- [39] R. Asmis, E.S. Wintergerst, Dehydroascorbic acid prevents apoptosis induced by oxidized low-density lipoprotein in human monocyte-derived macrophages, *Eur. J. Biochem.* 255 (1998) 147–155.
- [40] R. Asmis, J.G. Begley, Oxidized LDL promotes peroxide-mediated mitochondrial dysfunction and cell death in human macrophages—a caspase-3-independent pathway, *Circ. Res.* 92 (2003) E20–E29.
- [41] R. Kinscherf, R. Claus, M. Wagner, C. Gehrke, H. Kamencic, D. Hou, O. Nauen, W. Schmiedt, G. Kovacs, J. Pill, J. Metz, H.P. Deigner, Apoptosis caused by oxidized LDL is manganese superoxide dismutase and p53 dependent, *FASEB J.* 12 (1998) 461–467.
- [42] G. Lizard, C. Miguët, G. Bessède, S. Monier, S. Gueldry, D. Neel, P. Gambert, Impairment with various antioxidants of the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome *c* occurring during 7-ketocholesterol-induced apoptosis, *Free Radic. Biol. Med.* 28 (2000) 743–753.
- [43] L. Ryan, Y.C. O’Callaghan, N.M. O’Brien, Generation of an oxidative stress precedes caspase activation during 7 beta-hydroxycholesterol-induced apoptosis in U937 cells, *J. Biochem. Mol. Toxicol.* 18 (2004) 50–59.